II. Zong Does Not Utilize Aggregates

In the methods described by Zong, the starting material for high pressure treatment is not the pellet containing aggregated protein, but rather a soluble apo protein. Zong does form pellets, but does not use them in their high-pressure treatment. In the Materials and Methods section of the aforementioned paper (page, 12421, column 1), the authors state:

> The partially purified apoCPO was diluted to 100 μg/ml in refolding medium, which contained 20 mM potassium phosphate buffer, pH 6.5, 10 μg/ml bovine hemin (Sigma, Type 1), 1 mM GSSG, and 1 mM DTT. The solution was left to stand for 48 h at 4°C. ***Precipitated protein was then removed by centrifugation***. Small aliquots of the CPO ***supernatant*** fraction obtained in the preliminary reconstitution step were transferred to a series of 100 mM buffers .... All samples were pressurized to 207 MPa in 34.5 MPa increments, and then the temperature was lowered to –12°C in 2°C steps.

Furthermore, in previous processing steps, Zong took additional care to remove aggregated protein by centrifugation before attempting to obtain the holochloroperoxidase from the apoprotein found in soluble fractions. On page 12421, column 1, the authors state that:

> This procedure gave a urea-solubilized apoCPO protein that was about 70% pure, as judged from SDS-PAGE analysis. ***After centrifugation*** at 12000 g for 10 minutes, the ***soluble fraction was diluted*** ....

Thus, the paper of Zong teaches away from using pressure on aggregated proteins, in sharp contrast with the current invention. Zong took care to remove aggregates from samples prior to pressurization.

Furthermore, there is no evidence presented by Zong that the proteins to which high hydrostatic pressure was applied were indeed aggregated. In fact, Zong present data that argues ***against*** the presence of aggregated protein in the samples that they pressurized. Figure 6 shows changes in the absorbance ultraviolet spectra of the recombinant apoCPO constituted with heme

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during its incubation under high pressure. There is no change in the difference spectra in the wavelength range around 500-550 nm as a function of pressure or temperature. Under the conditions used in Figure 6, Zong found that the apoCPO bound heme to form the holoenzyme. If pressure treatment caused disaggregation, a large change in the difference spectra in this range would have been expected. Thus, there is no evidence that aggregated protein was present in the material that Zong treated with high pressure.

III. Applicants' Methods Utilize Aggregates

In the Advisory Action, the examiner stated that applicants' use of "comprising" leaves open the possibility that Zong anticipates. When called by the undersigned to explain this statement, the examiner indicated that the term "comprising" meant that applicants' claims could possibly include, prior to applicants' step (a), an additional step of aggregate removal (*e.g.*, centrifugation). If so, it was argued that the instant claims would read on Zong.

Applicants appreciate the examiner's clarification of this point, but respectfully disagree. For example, the term "mixture," which is used throughout the body of the claims, is defined in the preamble as "a mixture comprising aggregated protein." In light of this definition, it is believed that, to be used consistently, the term "mixture" in steps (a) and (b) must be interpreted as an "aggregate mixture." In addition, step (b) talks about a pressure sufficient to cause "deaggregation," which would make no sense if the aggregates had been removed prior to this step. Thus, Zong cannot anticipate applicants' claim 1, which uses aggregates in steps (a) and (b), as Zong ***eliminates*** the aggregated protein ***prior*** to any pressurization steps, as discussed above. Applicants respectfully request that the examiner reconsider his interpretation of the claims in light of these additional comments.

However, in order to make this point more clear, applicants have provided an amendment to the claims to further clarify that the mixture in steps (a) and (b) is an ***aggregate*** mixture. In addition, as noted by the examiner, the use of the term "aggregate" in step (d) is incorrect as aggregates are eliminated in step (b); a corresponding correction is provided. Clearly, this amendment is supported by the application as filed. In addition, there is no new search required as it is clear that the examiner has considered pressurization of aggregates during the prosecution:

> "The invention as described on page 6 makes use of a two-step pressure treatment of an **aggregated protein mixture.**" – paper #8, page 3 (emphasis added)
>
> "The ... claims [read] on a procedure that can be used on any **aggregated protein mixtures** to produce active protein of any type ...." – paper #12, page 3 (emphasis added)

Thus, applicants submit that the present amendments, though not believed necessary given a proper interpretation of the instant claims, more clearly specify the use of aggregated protein in steps (a) and (b), thereby eliminating any argument that Zong is anticipatory. Entry of the amendment is believed proper, and is respectfully requested.

IV. Conclusion

In light of the foregoing, it is respectfully submitted that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. Should Examiner Guttman have any questions, he is invited to contact the undersigned attorney at (512) 536-3184.

Respectfully submitted,

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